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USPT	l7 and adenovir$2	1	L8
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USPT	l4 same vector	59	L5
USPT	l3 same (inhibit$6 or suppress$6 or regress$6)	633	L4
USPT	l2 same (gene or dna or cdna or nucleic acid or polynucleotide)	781	L3
USPT	l1 same (cancer or tumor or neoplas$2)	1004	L2
USPT	p53	1760	L1

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Today's Date: 8/10/2000

DB Name	Query	Hit Count	Set Name
USPT	l1 and p53	1	L4
USPT	l1 and adenovir$2	1	L3
USPT	l1 and cmv	1	L2
USPT	6017524.pn.	1	L1

so long as the vector is capable of introducing the nucleic acid coding sequences into the genome of the targeted cell in a relatively stable fashion. By way of illustration, but not limitation, one can mention the following vectors, including N2A, LN, LNSX, Adenovirus and Adeno-associated virus.

ORPL:
Wills and Menzel, "Adenovirus Vectors for Gene Therapy of Cancer," Journal of Cellular Biochemistry, p. 204, Abstract # S216, Mar.-Apr. 1993.

ORPL:
Zhang et al., "Generation and Identification of Recombinant Adenovirus by Liposome-Mediated Transfection and PCR Analysis," BioTechniques, 15(5):868-872, 1993.

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L2: Entry 1 of 1 File: USPT Jan 25, 2000

DOCUMENT-IDENTIFIER: US 6017524 A
TITLE: Inhibiting the growth p53 deficient tumor cells by administering the p53 gene

BSPR:
The particular promoter that is employed to control the expression of the antisense RNA in a vector construct is not believed to be particularly crucial, so long as it is capable of expressing the antisense intron RNA in the targeted cell of a rate greater than 5 fold that of the gene to be inhibited. Thus, where a human cell is targeted, it will be preferred to position the antisense RNA coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human cellular or viral promoter. While the .beta.-actin promoter is preferred the invention is by no means limited to this promoter, and one may also mention by way of example promoters derived from RSV, N2A, LN, LNSX, LNSN, SV40, LNCX or CMV (Miller, et al., 1989; Hamtzoponlos, et al., 1989).

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L4: Entry 1 of 1 File: USPT Jan 25, 2000

DOCUMENT-IDENTIFIER: US 6017524 A
TITLE: Inhibiting the growth p53 deficient tumor cells by administering the p53 gene

ABPL:
Disclosed are methods and compositions for the selective manipulation of gene expression through the preparation of retroviral expression vectors for expressing antisense sequences, such as K-ras oncogene antisense sequences, or sequences encoding a desired product, such as wild type p53 sequences. Preferred retroviral vectors of the present invention incorporate the .beta.-actin promoter in a reverse orientation with respect to retroviral transcription. Preferred antisense RNA constructs of the present invention employ the use of antisense intron DNA corresponding to distinct intron regions of the gene whose expression is targeted for down-regulation. In an exemplary embodiment, a human lung cancer cell line (NCI-H460a) with a homozygous spontaneous K-ras mutation was transfected with a recombinant plasmid that synthesizes a genomic segment of K-ras in antisense orientation. Translation of the mutated K-ras mRNA was specifically inhibited, whereas expression of H-ras and N-ras was unchanged. A three-fold growth inhibition occurred in H460a cells when expression of the mutated ras p21 protein was down-regulated by antisense RNA and cells remained viable. The growth of H460a tumors in nu/nu mice was substantially reduced by expressed K-ras antisense RNA.

BSPR:
Another important "oncogene" is the gene encoding the p53 cellular protein. The p53 gene is one of the most common targets for genetic abnormalities in human tumors (Hollstein et al., 1991). For example, it has been reported that p53 mutations occur in all histological types of lung cancer at frequencies of about 75% in small cell lung cancer (SCLC) and about 50% in non small cell lung cancer (NSCLC) (Takahashi et al., 1991). Evidence suggests that p53 acts as a "tumor suppressor" gene, and its inactivation through mutation can lead to oncogenic development. In fact, a predominance of G to T transversions in p53 and ras mutations in lung cancer, as well as epidemiological data, supports a close association between smoking and p53 mutations in NSCLC have suggested that p53 is a candidate for molecular targets of genetic damage caused by cigarette smoke (Zakut-Houri et al., 1985).

BSPR:
One approach that has been suggested as a means of treatment of such tumors is the introduction of so-called "wild-type" or non-mutated p53 (wt-p53) into affected cells, e.g., through the use of retroviral vectors which encode the wild type protein (Takahashi et al., 1992; Lee et al., EP appl. publ. 0 475 623 A1). The vectors proposed by these individuals included a wt-p53 genes wherein the direction of transcription of the encoded wt-p53 was in the same orientation as that of the retroviral long terminal repeats (LTRs). Unfortunately, in studies conducted by the present inventors reported hereinbelow, the ability of retroviral wt-p53 constructs prepared having such an orientation to suppress tumor growth was found to be less than optimal. Presumably, this shortcoming is the result of poor expression of the wt-p53 gene in the target cells.

BSPR:
In one specific embodiment, the invention concerns vector constructs for introducing wild type p53 genes (wt-p53) into affected target cells suspected of having mutant p53 genes. These embodiments involve the preparation of a gene expression unit wherein the wt-p53 gene is placed under the control of the .beta.-actin promoter, and the unit is positioned in a reverse orientation into a retroviral vector.

BSPR:
While aspects of the invention are exemplified through the use of wt-p53 constructs, and their use in cancer treatment, it is proposed that the invention is generally applicable to any situation where one desires to achieve high level expression of a recombinant protein in a target or host cell through the use of a retroviral expression vector. This could, for example, involve the use of a gene encoding a recombinant protein that confers a particular trait, such as the use of wt-p53 to "replace" a trait that has been lost due to mutation, or could be used to introduce protein-encoding genes that one desires to use for preparing proteins for other purposes, such as in recombinant protein production procedures. While the nature of the gene introduced is not critical to broader aspects of the invention, it should be mentioned that in the context of cancer treatment modalities, a particular example in addition to p53 replacement that is contemplated by the inventors is the introduction of the retinoblastoma gene (rb).

DRPR:
FIG. 2B. An RNA PCR analysis was done to compare the level of K-ras message in H460a and H460a transfectants. As a control, a portion of p53 gene was co-amplified with p53 specific primer which served as an internal control.

DRPR:
FIG. 2C and FIG. 2D. H-ras and N-ras specific amplimers were used to quantitate H-ras/N-ras RNA in the transfectants and parental cell lines. p53 gene amplification is shown as an internal control.

DRPR:
FIG. 13 Growth curves are shown for 10.sup.4 cells/well seeded in 12 well plates. H322a cells were infected by incubation 0.5 m of viral supernatant stock from either LNSX, DC, LNSX-p53 or DC-p53 (10.sup.6 CFU/ml) on 2 consecutive days in the presence of 8 .mu.g/ml of polybrene. The parental H322a cells served as a control. Cells were not selected with G418. Cells were counted daily. The mean .+-.SE is shown for three replicates.

DRPR:
FIG. 14 Growth curves are shown for 10.sup.4 cells/well seeded in 12 well plates. H460a cells were infected by incubation 0.5 m of viral supernatant stock from either LNSX, DC, LNSX-p53 or DC-p53 (10.sup.6 CFU/ml) in the presence of 8 .mu.g/ml of polybrene. The parental H322a cells served as a control. Cells were not selected with G418. Cells were counted daily. The mean .+-.SE is shown for three replicates.

DEPR:
The p53 gene is the most frequently mutated gene yet identified in human cancers. It is mutated in over 50% of human NSCLC (Hollestein et al., 1991). The p53 gene encodes a 375-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B (Lane et al., 1990). Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. The wildtype p53 gene may directly suppress uncontrolled cell growth or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wildtype p53 may contribute to transformation. However, some studies indicate that the presence of mutant p53 may be necessary for full expression of the transforming potential of the gene. Mutations of p53 are common in a wide spectrum of tumors (Bressac et al., 1990; Dolcetti et al., 1990; Rodrigues et al., 1990; Nigro et al., 1989); they occur in both NSCLC and SCLC cell lines and fresh tumors (Nigro et al., 1989; Takahashi et al., 1989).

DEPR:
cDNA synthesis was carried out in a total volume of 20 .mu.l containing 5 .mu.g of total RNA and oligo (dT) as a primer (Becker-Andre, et al., 1989). A portion of the cDNA corresponding to the first and second exons was amplified to monitor the level of endogenous K-ras mRNA (FIG. 2A) using KA12 and KB61 amplimers. Denaturation, annealing, and extension were done at 92.degree. C. for 1 min, 51.degree. C. for 1 min and 74.degree. C. for 1 min, respectively. However, annealing temperatures for N-ras and H-ras were 44.degree. C. and 42.degree. C., respectively. In addition, two amplimers were also used in the same reaction mixture to amplify a 118-bp fragment of the p53 gene as an internal control. PCR products were either transferred onto a membrane and hybridized with .sup.32 P labelled cDNA probe or alternatively, there were directly labelled during the last cycle of amplification by adding 1 uCi of .sup.32 P dCTP. The labelled PCR products were loaded on an 8% nondenaturing polyacrylamide gel. The gel was photographed after ethidium bromide staining, dried, and exposed to x-ray film overnight at -80.degree. C.

DEPR:
The effect of AS RNA on the specific production of mature endogenous K-ras mRNA was analyzed by cDNA PCR (FIG. 2A). cDNA synthesized from the total RNA (Chomczymsky, et al., 1987) was subjected to PCR amplification using amplimers corresponding to the 5'-end of the first exon and the 3'-end of the second exon (FIG. 2A). Because the AS RNA was generated only from a second and third exon of the K-ras gene, PCR amplified cDNA represented the level of endogenous K-ras mRNA. A 246-bp amplified DNA fragment was labelled by .sup.32 P dCTP and subsequently analyzed by polyacrylamide gel electrophoresis. In addition, a 118-bp segment of endogenous p53 cDNA was co-amplified in the same reaction mixture using p53 specific amplimers to serve as an internal control for the PCR.

DEPR:
Results showed that H460a cells, clones expressing S RNA, and the Calu-1 cell line expressed K-ras mRNA, as evidenced by the presence of a high level of amplification of the 246-bp cDNA product (FIG. 2B). H460a clones expressing AS RNA showed very little amplification, and cellular K-ras mRNA synthesis appeared to be completely inhibited (FIG. 2B, lanes 5 and 6). In contrast, the endogenous p53 expression remained unaffected. This prompted us to investigate the level of expression for other ras genes in these clones. We employed the same cDNA PCR methodology to analyze the N-ras and H-ras mRNA level using N-ras and H-ras-specific oligonucleotides as amplimers. A steady state level of H-ras and N-ras gene expression was observed, but no obvious change either in Apr-1-neo AS or Apr-1-neo S transfectants was noticed (FIG. 2C and FIG. 2D). The p53 gene expression serving as a control in these experiments remained unaffected. Thus, inhibition of K-ras expression by our AS RNA construct is specific.

DEPR:
This example is provided to demonstrate a protocol for administering and assessing the efficacy and toxicity of the intralesional administration of retroviral constructs containing antisense (AS) K-ras (for tumors with mutated K-ras) and wildtype p53 (wtp53) (for tumors with mutated or deleted p53) into residual endobronchial NSCLC which obstructs a bronchus and which is refractory to conventional therapy.

DEPR:
The 2 Kb K-ras fragment (genomic exons 2 and 3) with a .beta.-actin promoter was cloned into the LNSX retroviral vectors in both orientations. The p53 cDNA with its .beta.-actin promoter was cloned into the LNSX retroviral vectors in both orientations. Both the LNSX-AS-K-ras and the N2A-AS-K-ras have been successfully packaged in the GP+envAm12 packaging cell line. Initial titers ranged up to 10.sup.4. By using a "ping-pong" technique, the titer of the LNSX-AS-K-ras supernatant was increased to 5.times.10.sup.6. In this technique, supernatants from the GP+envAm12 packaging cell line were used to transduce the ecotropic packaging cell line .PSI.2 (Mann et al., 1983). Supernatants from this transduction were

used again to transduce GP+envAm12. Both constructs were then transduced into H460a cells. Specific expression of K-ras AS RNA was shown by slot blot analysis using vector only negative controls and a .beta.-actin probe for a loading control. Western blotting studies showed that expression of the K-ras p21 protein was specifically reduced. Next the effect of multiple cycles of transduction on transduction efficiency was assessed. Transduction efficiency was assessed on a functional level (FIG. 11). H460a cells were transduced with either LNSX or LNSX-AS-K-ras daily for 4 consecutive days. Cells grew for 7 days without selection.

DEPR:
The p53 gene is the most commonly altered gene yet described in human cancers. To study this gene, a cell culture model system of cell lines varying in p53 expression was established. The H322a lung adenocarcinoma cell line expresses the mutant p53 protein as shown by the presence of high levels of endogenous p53 mRNA and phosphorylated protein. We showed that the H322a cell line has a G:T transversion at codon 248 (Arg to Leu) with absence of the wildtype allele. The H358a cell line has a homozygous p53 deletion. The H460a and H226b cell lines are homozygous for the wildtype p53. Expression vectors for sense (S-p53) and antisense p53 (AS-p53) cDNA with a .beta.-actin promoter were constructed to study the effect of wtp53 expressed in lung cancer cells with mutant or deleted p53 and the effects of reducing wildtype and mutant p53 expression.(Mukhopadhyay et al., 1991)

DEPR:
Stable transfectants of p53 mutant cells (H322a) or deleted p53 (H358) expressing S-p53 could not be rescued. Failure to isolate colonies expressing sense p53 RNA in cells with homozygous mutant or deleted alleles shows that wtp53 can suppress transformation in cancer cells expressing a mutant p53 or having a homozygous p53 deletion.

DEPR:
In general, transfection with AS-p53 reduced colony formation (10-fold) by cells with endogenous mutant p53. This indicates that expression of mutant p53 contributes to the transformed phenotype. As expected, cells with wtp53 (H226b) showed increased tumorigenicity when transfected with AS-p53. The H226b cells expressing AS-p53 grow significantly more rapidly in nu/nu mice than the cells transfected with the control plasmid. This indicates that elimination of the wtp53 gene product enhances features of the malignant phenotype.

DEPR:
The inventors studies showed that wtp53 is dominant and can suppress the malignant phenotype in cells with mutant or deleted p53. The presence of the mutant p53 confers transforming potential to the gene product, which can be suppressed by AS-p53. Thus, in cancer cells both the absence of wtp53 and the presence of certain p53 mutations may enhance the malignant phenotype.

DEPR:
The retroviral vector construct contains p53 cDNA with its .beta.-actin promoter inserted into the LNSX vector (Miller et al., 1989; Palmer et al., 1987) in a reverse orientation, in essentially the same manner as described for the p21 AS embodiments.

DEPR:
The LNSX-p53 and the DC-p53 were transduced into H322a (mutant p53), H358a (deleted p53), and H460a (wt p53). H322a cells that underwent one cycle of infection with the wtp53 construct but without G418 selection had an over 3-fold reduction in proliferation compared to cells that received either the unmodified vector or no treatment. Two cycles of transduction without G418 selection resulted in a 5-fold reduction in proliferation (FIG. 13). A similar result was observed for the H358a cell line when transduced with LNSX-p53. The proliferation of the H460a cell line which has a wildtype p53 was not altered by transduction with any of the p53 retroviral constructs (FIG. 14). Thus, retroviral mediated gene transfer of wtp53 into human lung cancer cells with deleted or mutated p53 significantly reduces the proliferation of those cells. The expression of the mutated p53 protein is uniform in cultured cell lines as detected by immunohistochemistry. In fresh lung tumors that express high levels of p53 protein, expression is detected in >90% of cells.

DEPR:
Injections will be circumferential and will be intratumor and submucosal. The AS-K-ras supernatant will be used for K-ras mutations and the p53 supernatant will be used for p53 mutations. The injections will be repeated daily for five consecutive days. The treatment will be repeated monthly.

DEPU:
Bressac B, Galvin K M, Liang T J, Isselbacher K J, Wands J R. Abnormal structure and expression of p53 gene in human heptatocellular carcinoma. Proc Natl Acad Sci USA 1990; 87:1973-1977.

DEPU:
Casson A G, Mukhopadhyay T, Cleary K R, Ro J Y, Levin B, Roth J A. p53 gene mutations in Barrett's epithelium and esophageal cancer. Cancer Res 1991; 51:4495-4499.

DEPU:
Chen P-L, Chen Y, Bookstein R, Lee W-H. Genetic mechanisms of tumor suppression by the human p53 gene. Science 1990; 250:1576-1580.

DEPU:
Dolcetti R, Maestro R, Feriotto G, Pelucchi S, Rizzo S, Boiocchi M. p53 genetic abnormalities in human squamous cell carcinomas of the larynx. Oncogene 1990; 6:44-45.

DEPU:
Hollstein M, Sidransky D, Vogelstein B, Harris C C. p53

mutations in human cancers. Science 1991; 253:49-53.

DEPU:
Mukhopadhyay T, Cavender A C, Branch C D, Roth J A. Expression and regulation of wild type p53 gene (wtp53) in human non-small cell lung cancer (NSCLC) cell lines carrying normal or mutated p53 gene. J Cell Biochem 1991; Suppl 15F:22.

DEPU:
Nigro J M, Baker S J, Preisinger A C, et al. Mutations in the p53 gene occur in diverse human tumor types. Nature 1989; 342:705-708.

DEPU:
Rodrigues N R, Rowan A, Smith M E F, et al. p53 mutations in colorectal cancer. Proc Natl Acad Sci USA 1990; 87:7555-7559.

CLPR:
1. A retroviral expression vector comprising a gene expression unit which comprises a wild-type p53 gene under the control of a .beta.-actin promoter, the gene expression unit being positioned to effect transcription of the gene in an orientation opposite that of retroviral transcription.

CLPR:
8. A method for the preparation of a retroviral expression vector comprising constructing a gene expression unit which comprises a wild-type p53 gene placed under the control of a .beta.-actin promoter, and positioning the gene expression unit into a retroviral vector in an orientation opposite that of retroviral transcription.

CLPR:
9. A method for treating cancer in a human patient comprising directly introducing into a p53-deficient tumor cell of the patient a retroviral expression vector dispersed in a pharmaceutical diluent, wherein said expression vector comprises a gene expression unit which comprises a wild-type p53 gene under the control of a .beta.-actin promoter, the gene expression unit being positioned to effect transcription of the gene in an orientation opposite that of retroviral transcription, and wherein expression of p53 by said expression vector is effective to inhibit the growth of said tumor cell.

ORPL:
Casey et al., "Growth Suppression of Human Breast Cancer Cells by the Introduction of a Wide-Type p53 Gene," Oncogene, 6:1791-1797, 1991.

ORPL:
Casson et al., "p53 Gene Mutations in Barrett's Epithelium and Esophageal Cancer," Cancer Research, 51:4495-4499, 1991.

ORPL:
Chen et al., "Genetic Mechanisms of Tumor Suppression by the Human p53 Gene," Science, 250:1576-1580, 1990.

ORPL:
Chen et al., "Expression of Wild-Type p53 in Human A673 Cells Suppresses Tumorigenicity but Not Growth Rate," Oncogene, 6:1799-1805, 1991.

ORPL:
Gusterson et al., "Expression of p53 in Premalignant and Malignant Squamous Epithelium," Oncogene, 6:1785-1798, 1991.

ORPL:
Takahashi et al., "Wild-Type but Not Mutant p53 Suppresses the Growth of Human Lung Cancer Cells Bearing Multiple Genetic Lesions," Cancer Research, 52:2340-2343, 1992.

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L3: Entry 1 of 1 File: USPT Jan 25, 2000

DOCUMENT-IDENTIFIER: US 6017524 A
TITLE: Inhibiting the growth p53 deficient tumor cells by administering the p53 gene

BSPR:
Antisense technology has also been applied in attempts to inhibit the expression of various oncogenes. For example, Kasid, et al., 1989, report the preparation of recombinant vector construct employing Craf-1 cDNA fragments in an antisense orientation, brought under the control of an adenovirus 2 late promoter. These authors report that the introduction of this recombinant construct into a human squamous carcinoma resulted in a greatly reduced tumorigenic potential relative to cells transfected with control sense transfectants. Similarly, Prochownik, et al., 1988, have reported the use of Cmyc antisense constructs to accelerate differentiation and inhibit G.sub.1 progression in Friend Murine Erythroleukemia cells. In contrast, Khokha, et al., 1989, discloses the use of antisense RNAs to confer oncogenicity on 3T3 cells, through the use of antisense RNA to reduce murine tissue inhibitor or metalloproteinases levels.

BSPR:
In broader aspects of the invention, a preferred approach will involve the preparation of retroviral vectors which incorporate nucleic acid sequences encoding the desired construct, once introduced into the cells to be treated, preferably, these sequences are stably integrated into the genome of the cell. One example of such of vector construct would be a replication defective retrovirus, such as LNSX, LN or N2A, that is made infective by appropriate packaging, such as by GPtenvAM12 cells. Although the retrovirus would inhibit the growth of the tumor, the expression of the antisense construct in non-tumor cells would be essentially harmless where one prepares a retrovirus construct which encode distinct antisense intron RNA in accordance with the present invention. In addition to retroviruses, it is contemplated that other vectors can be employed, including adenovirus, adeno-associated virus, or vaccinia viruses (Hermonat, et al., 1984; Karlsson, et al., 1985; Mason, et al., 1990).

DEPR:
The particular vector which one employs for introduction of antisense intron coding sequences is not believed to be particularly crucial to the practice of the present invention,